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During the first year of the grant, we obtained sufficient amounts of the T-cell receptor (TCR) for crystallization experiments. This was achieved by protein engineering and by protein refolding experiments. The refolded TCR is now readily obtained in large amounts. The refolded TCR binds to its specific peptide/HLA-A2 complex as detected by native polyacrylamide gel electrophoresis. The ternary complex of TCR/HLA-A2/peptide forms large moderately-well diffracting crystals. We have collected a 3.3 Angstrom dataset and are in the process of determining the structure of the complex to the limit of our data. This structural determination of the T-cell receptor/HLA-A2/peptide complex should allow a detailed understanding of this central molecular recognition event in the immune system.

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Annual Report (1 Sep 1994 - 31 Aug 1995)

Structural Analysis of the Human T-cell Receptor/ HLA-A2/Peptide Complex

Introduction

Cytotoxic T-lymphocytes (CTL) play a vital role in the immune response to antigen by their ability to distinguish between self and foreign cells. The basis for this differential recognition is the specific binding of T-cell antigen receptors (TCR) to antigens in the form of peptides bound to human leukocyte antigen (HLA) proteins at the cell surface. The antigenic peptides are derived proteolytically from proteins synthesized within the cell. Many tumors have been shown to express tumor-specific antigens whose peptides can label tumor cells as being "foreign", thus promoting the specific lysis of the tumor cell by CTLs. Little is known of the molecular interaction or recognition between a T-cell receptor and an HLA protein/peptide complex that leads to CTL responses.

In the first year of this grant, we have begun the determination of the x-ray structure of a TCR/HLA-A2/peptide complex that should yield an understanding of the recognition that is required for specific T-cell responses and should aid in the development of immunotherapies using tumor-specific CTLs to control tumor growth.

To obtain sufficient amounts of protein for crystallization, the human TCR and HLA-A2 proteins are being produced in bacteria. Abundant HLA-A2 complexed with single peptides has been available from previous work (1). Refolding experiments with the TCR obtained from bacterial expression have been successful and ample TCR protein is now available. We have prepared x-ray quality crystals of the TCR/HLA-A2/peptide complex and have collected a dataset to a resolution limit of 3.3 Å. We are in the process of solving the TCR/HLA-A2 structure by

molecular replacement techniques using the HLA-A2 structure (2) and the published structures of domains of a murine TCR (3, 4).

Results

The first-year task for this project was to obtain sufficient amounts of the TCR protein, since relatively large amounts of protein are needed for crystallization experiments. Our approach was to overexpress the alpha and beta subunits of the TCR in bacteria, to isolate the overexpressed, but insoluble proteins, then to refold the subunits into their native state. The TCR used is the anti-tax peptide (LLFGYPVYV) receptor that is restricted to HLA-A2 as described in the application.

Refolding by dialysis.

The denatured alpha and beta subunits were separately diluted into a buffer containing 100 mM Tris-HCl, 400 mM l-arginine-HCl, 5 M urea, 2 mM EDTA, pH 8.3 to a protein concentration of 2 uM. This was dialyzed against the same buffer but lacking the urea and then dialyzed against 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM DTT, pH 7.5. The dilute proteins were concentrated by in a stirred ultrafiltration cell (Amicon). Since the TCR found in human cells has a disulfide bond between the alpha and beta subunits, it is possible that this bond is required for the TCR to fold, to be active , and/or to signal. Therefore, we initially produced a refolded TCR with this inter-subunit disulfide bond. The beta protein was treated with Ellman's reagent (Sigma) to derivatize the free cysteine at the C-terminus of the protein. Then both alpha and beta were again dialyzed against 20 mM Tris/150 mM NaCl, pH 7.5, to remove the DTT and the Ellman's reagent. After re-concentration, alpha and beta were mixed at a 1:1 molar ratio to form a disulfide bond between the free C-terminal cysteine on the alpha subunit and the

derivatized, activated C-terminal cysteine on the beta subunit. The TCR protein was again dialyzed and concentrated and used directly for binding studies.

Binding Assay Using Native Polyacrylamide Gels

The refolded TCR was tested for its ability to bind its specific HLA-A2/tax peptide complex. Upon mixing the TCR and HLA-A2/tax proteins and electrophoresing the mixture on polyacrylamide native gels, a new band appeared which differed in mobility from the bands observed when either the TCR or the HLA-A2 protein were electrophoresed alone. Native gels were 15% acrylamide and used standard Tris/glycine buffers without SDS. The new band appeared to be the complex of TCR and HLA-A2. No new band appeared when the peptide bound to HLA-A2 was an altered tax peptide having position 4 (G -> K) and position 8 (Y -> K) mutations. The complex band was isolated from the gel and re-electrophoresed on an SDS denaturing gel. The protein bands for the two HLA-A2 subunits appeared as well as a 55 kD band (unreduced sample buffer) or an intense 28 kD band (reduced sample buffer). This is evidence that the TCR and HLA-A2 were forming a complex. The denatured TCR alpha and beta subunits have the same mobility on SDS gels, thus the single intense band seen using reducing sample buffer. The marked change in mobility upon reduction of disulfide bonds indicated that the interchain disulfide was present. The dialysis/Ellman's procedure for preparing disulfide-bonded TCR protein was successful in that it yielded protein that specifically bound HLA-A2 complexed with the tax peptide, but the procedure did not yield sufficient purified protein for crystallizations.

The Interchain Disulfide is Not Necessary for Binding.

The cysteines involved in the interchain disulfide bond may lower yields of properly folded protein, since during refolding unpaired cysteines can catalyze disulfide exchange to form non-native disulphide bonds or become oxidatively damaged, especially in the presence of protein denaturant. To test whether the

TCR interchain disulfide bond was required for complex formation, iodoacetamide (IAA) was added to the refolded alpha and beta subunits. IAA covalently modifies free cysteines, making them no longer reactive in disulfide bond formation. After mixing the IAA-treated TCR subunits, no disulfide bond was evident by SDS gel electrophoresis (reduced and nonreduced). A new complex band still appeared on native gels after mixing the IAA-treated TCR subunits and the HLA-A2/tax protein. This implied that the disulfide bond between alpha and beta is not necessary for binding to HLA-A2. To remove the unneeded cysteines, we moved the stop codons in the plasmid constructions for both the alpha and beta proteins. For alpha, the single C-terminal cysteine was replaced by a stop codon by polymerase chain reaction mediated mutation (E. Tienhoven, DNG, DCW). For beta, the single C-terminal cysteine was replaced by a stop codon in an analogous manner (J.-S. Kim, DNG, DCW). In addition for beta, the free cysteine within the constant domain was replaced by an alanine residue (3). These stop codon changes also altered the lengths of both alpha and beta, so that they can now be distinguished by their differing mobilities on SDS denaturing gels.

Refolding by Dilution

Our current optimized procedure for refolding the anti-tax TCR by dilution is as follows. Alpha and beta inclusion body protein is prepared in 50 mM MES, 8 M urea, 2 mM NaEDTA, 0.1 mM DTT at approximately 10-20 mg/ml as determined by a dye-binding assay (Biorad) (ref. 1). Equal amounts of alpha and of beta (~30 mg) are diluted into 6 M guanidine-HCl, 10 mM NaAcetate, 2 mM EDTA pH 4.5 and kept at room temperature. A liter of refolding buffer is prepared consisting of 100 mM Tris-HCl, 400 mM l-arginine-HCl, 2 mM NaEDTA, 6.3 mM cysteamine, 3.7 mM cystamine and adjusted to pH 8.5 at room temperature. The refolding buffer is brought to 10°C. The alpha/beta guanidine solution is injected into the refolding buffer through a #27 needle while stirring the refolding buffer

vigorously. The refolding TCR solution is kept at 10°C for 24 h. It is then dialyzed against 10 liters of 100 mM urea at 4°C for 24 h and then against a new 10 liters of 10 mM Tris-HCl, 100 mM urea, pH 7.5 for 24 h more. The dialyzed protein solution is loaded on to a column of DE52 anion exchange resin (Whatman) in 10 mM Tris-HCl, pH 7.5 and the protein is eluted into ~50 ml with 10 mM Tris-HCl, 300 mM NaCl, pH 7.5. After concentration in a Centriprep-30 (Amicon), the TCR is purified on a Superdex-75 gel filtration column (Pharmacia). The purified protein is concentrated to 50-75 mg/ml and buffer-exchanged to 10 mM Tris-HCl, pH 7.5.

Crystallization and Data Collection

The TCR (75 mg/ml) and HLA-A2/tax (12 mg/ml) were mixed at a 1:1 molar ratio and set up in a hanging drop vapor diffusion crystallization experiment. Crystals appeared in 10% polyethylene glycol, 100 mM MgAcetate, 50 mM NaCacodylate, pH 6.5. These crystals were reseeded (5) and large (200 x 200 x 100 microns) crystals were obtained. The larger crystals were mounted in an glass capillary tube for diffraction. Diffraction was observed to about 3.5 Å resolution. Crystals were placed into the above buffer plus 20% glycerol and frozen at -160°C . Diffraction was observed to about 3.3 Å and a complete dataset was measured to 3.5 Å (60% complete 3.3-3.4 Å). The spacegroup is C2, with cell dimensions, a=228.2, b=49.6, c=94.7 Å, and beta=91.3°.

Conclusions

In the first year of the project we have refolded a TCR and showed that it binds to its specific peptide/HLA-A2 complex. We have an ample supply of protein for crystallographic studies. At present we are solving the structure of the TCR/HLA-A2 complex to 3.3 Å resolution. This is moderate resolution and we will attempt to obtain higher resolution data. With the laboratory rotating anode x-

ray source, the limit of diffraction of these crystals appears to be ~3.3 Å, though we are continuing to search for a modified set of conditions for freezing the crystals (buffers, temperature, protocol, etc.) that will lead to higher resolution data. We have applied for time at the Cornell High Energy Synchrotron Source (CHESS) to use the high intensity x-rays generated there to obtain better data from our present crystals. We continue to experiment in finding new crystal forms that will allow higher resolution data to be collected in our laboratory or elsewhere.

It is possible that the phases yielded by the molecular replacement solution using the HLA-A2/peptide structure will not yield an interpretable electron density map, especially at the current resolution of our data. Several strategies to overcome this are planned. (a) Heavy atom derivative data will be obtained to provide phases through standard crystallographic methods. The unique free cysteine in the constant region of beta will be engineered back into the protein for reacting with a mercury compound. (b) Seleno-methionine will be incorporated into one or more or the four subunits making up the TCR/HLA-A2 complex to provide a heavy atom derivative and anomalous scatterering for phase improvement. The large seleno-methionine residues also act as "landmarks" and aid in the tracing of the polypeptide chain through experimental electron density. (c) Obtaining data from a second crystal form would allow cross-crystal averaging to improve the electron density map to a more interpretable quality.

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